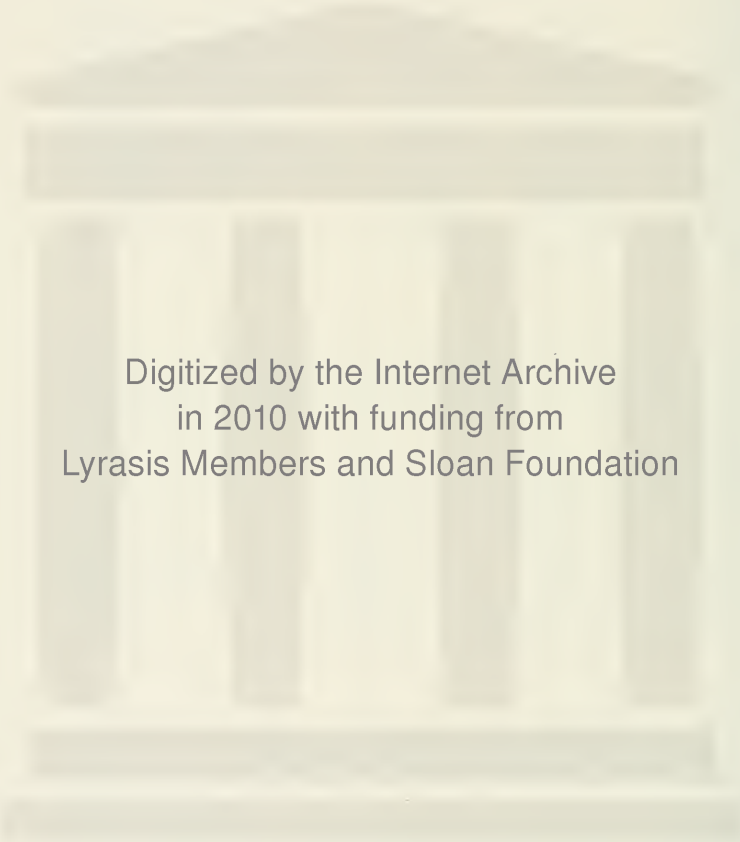

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Isolation and Identification of The Oak Wilt Fungus

by H. L. Barnett

WEST VIRGINIA UNIVERSITY AGRICULTURAL EXPERIMENT STATION

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Isolation and Identification of The Oak Wilt Fungus

H. L. BARNETT

The known range and distribution of oak wilt has been greatly extended during the last few years. During this time there has been a great increase in the public interest and in research efforts to determine its present distribution and to develop effective control measures. This has resulted in a greater number of trees being sampled and a greater demand on laboratory space and help for isolation of the oak wilt fungus (*Endoconidiophora fagacearum* Bretz). Some workers with little experience in mycology have been called upon to do the isolation and detailed examination of the fungi obtained. Since the only certain method for determining oak wilt is by the isolation of the pathogen, the need for routine isolation work will not end with the completion of the surveys. Isolation from oak trees is an essential part of inoculation and transmission studies as well as other phases of the research program. For these reasons it is highly desirable to use a medium and technique which would permit easy, quick, and accurate identification of the oak wilt pathogen.

The usual methods and media used in routine isolation of most fungi were found to be unsatisfactory. The use of malt extract or potato-glucose agar gave heavy mycelial growth, but the typical conidiophores producing endoconidia could seldom be found until ten days to two weeks after isolation.

The purpose of this bulletin is to describe the method of isolation, the special medium and the identifying cultural characteristics of the fungus used successfully in the oak wilt research laboratory at West Virginia University. It is believed that this information will be helpful to others who desire quick determination of oak wilt.

Methods of Isolation

Isolation of the oak wilt fungus usually can be accomplished easily from samples of an oak tree showing wilt symptoms, provided the bark has not dried out and the wood has not been invaded by

secondary fungi. The best samples are from branches about 1 inch in diameter, with the inner bark still fresh and green. If possible, samples should be taken from at least six branches from different parts of the tree. Slab samples from the trunk of dying trees, including the last few annual rings with attached bark are satisfactory. In fact, slab samples from the trunk or roots of dying trees may yield the oak wilt fungus, whereas branch samples may give only secondary fungi.

Samples should be kept under refrigeration, if possible, until isolations are made. Branch samples should first be washed with a brush and cool water to remove the dust and grit. The bark (at least the outer bark) is then stripped off with a knife (Fig. 1).[1] Surface sterilization of both the knife and branch may then be accomplished by dipping in 95 per cent alcohol and flaming (Fig. 2).

Some workers prefer to surface sterilize in this manner before removing the bark, allowing the bark to serve as an insulator protecting the wood from the heat. However, it is felt that removal of bark before the alcohol dip gives more complete surface sterilization. Results of several trials show that neither the alcohol nor the flame on the bare wood has any effect on the fungus in the wood.

Small chips of wood are cut through the two or three outer annual rings and with sterile forceps the chips are pushed down into the agar medium in Petri dishes (Fig. 3). Slab samples may be treated in a similar way but must be split to convenient size for handling.

There is considerable variation in the frequency with which the fungus is obtained from different branches of an oak wilt tree and even chips from the same branch. In some cases all or nearly all of the chips from a tree yielded the oak wilt fungus, whereas only a few chips of fifty to sixty from other trees were positive. It is not unusual to isolate the fungus from only one branch, the chips from other branches remaining sterile. One tree was proved to have oak wilt only after the third set of samples was taken and cultured. The presence of certain other fungi in the wood soon after the branches die almost totally excludes the growth of *E. fagacearum* in isolation plates. It is believed that a careful selection of good, fresh samples from living portions of the suspected tree is the best solution to this problem.

Isolation Media

The selection and use of a medium favorable for quick production of endoconidia or of other characteristic structures is quite important.

[1]Photographs in Figures 1, 2, and 3 were taken by William Strunk, West Virginia State Conservation Commission.

FIGURE 1. REMOVAL OF BARK from the oak sample reduces the chances of contamination from organisms on the bark.

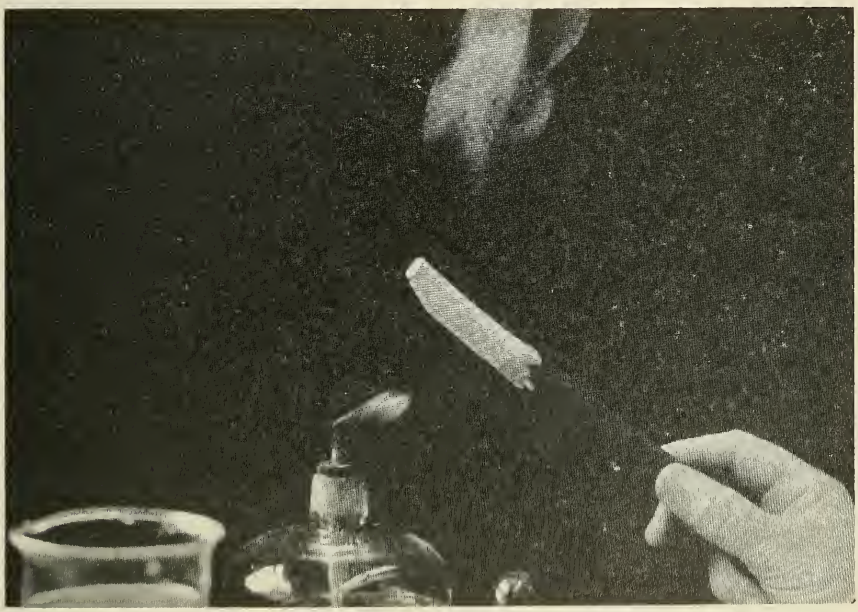

FIGURE 2. A DIP in 95 per cent alcohol and flaming sterilizes the surface.

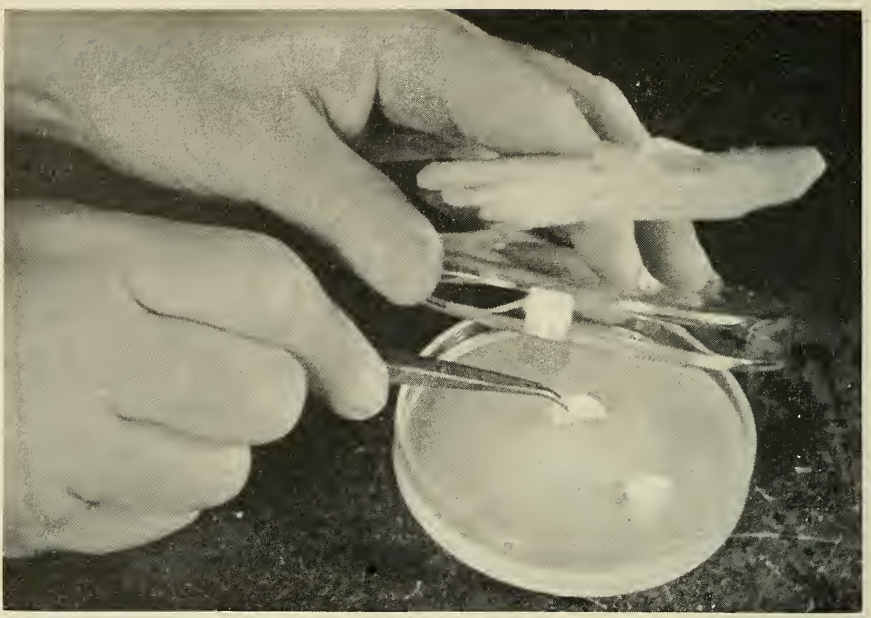

FIGURE 3. CHIPS OF WOOD through the outer two or three annual rings are cut with a sterile knife and placed in agar medium in a Petri dish.

A number of natural media, such as malt extract, potato-glucose, acorn and chestnut agars, are good for growth of the oak wilt fungus, but the production of conidia on these media may be delayed. When malt extract is used as an isolation medium, positive identification usually cannot be made for eight to ten days. It also is necessary to make mounts for microscopic eximination to determine whether endoconidia are present. When large numbers of samples are being cultured, it is highly desirable to reduce both the time and the amount of work involved. It also is desirable to use a synthetic or semisynthetic medium that can be reproduced accurately.

In 1951 it was discovered that *E. fagacearum* sporulated within twenty-four to forty-eight hours when a bit of mycelium was submerged in liquid media. Chips from oak wilt trees when placed directly in liquid media produced mycelium and endoconidia within four or five days. A number of media are satisfactory. It is believed to be a certain method of identification, for few fungi are able to sporulate when submerged in liquid. This method has been described briefly.[2]

Although this technique was successful in reducing the time required for identification, it still required either the use of agar medium

[2]Barnett, H. L., "A Rapid Method for Determining Oak Wilt." **Phytopathology**, 42:75 (1952).

for isolation and transfer of mycelium to liquid, or the use of a large number of tubes of liquid medium so that one freshly cut oak chip could be placed in each tube. Preparation of a microscope slide also was necessary for identification. The time and material required are the great disadvantages of this method, even though it is otherwise satisfactory.

It has been observed that the oak wilt fungus produces conidiophores and endoconidia quickly when spores are placed on agar medium. When non-sporulating mycelium is placed on agar mycelial growth continues, but few conidia are produced immediately. Since the fungus is believed to exist in the vessels of the oak tree mainly in the form of conidia, early growth following germination of spores in the chips should produce typical conidiophores and endoconidia. This was found to be true. Most isolates of *E. fagacearum* on favorable media immediately produce a few conidiophores and conidia on or beneath the surface of the agar near the oak chip. This early sporulation is followed by a period of vegetative growth in which few spores are formed. After ten to twelve days conidia are produced in great abundance. To be satisfactory, the isolation medium must be favorable to the early sporulation.

A search was made for a semisynthetic agar medium favorable for early sporulation and on which the mycelium could be examined directly with the compound microscope. Seventeen nitrogen sources were tested in a medium containing varying amounts of glucose, sucrose and maltose as the carbon sources. The best medium for this purpose was found to be glucose-phenylalanine agar. A brief description of this medium and the method used has been published.[3] The medium has been modified only slightly. Its composition is as follows:

Glucose	3 g.
Phenylalanine	0.5 g.
KH_2PO_4	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
Micro element solution[4]	2 ml.
Biotin	5 μg.
Thiamine hydrochloride	100 μg.
Distilled water	1 l.
Agar	20 g.

[3]Barnett, H. L., "A New Method for Determination of the Oak Wilt Fungus." (Abst.) **Phytopathology**, 42:1-2 (1952).

[4]A stock solution of micro elements may be made by using $Fe(NO_3) \cdot 9H_2O$, 723 mg.; $ZnSO_4 \cdot 7H_2O$, 440 mg.; $MnSO_4 \cdot 4H_2O$, 203 mg. Dissolve in 600 ml. of distilled water, add sufficient C.P. sulfuric acid to yield a clear solution, and make up the volume to 1 liter with distilled water.

An initial pH of 5.5 to 6 is satisfactory. The medium is autoclaved at fifteen pounds pressure for fifteen minutes and poured into Petri dishes.

This medium differs in two principal ways from the usual general purpose media. It is low in sugar, and phenylalanine is the sole source of nitrogen. Both features tend to slow down the growth of some of the common air contaminants. After inoculation a temperature of 20° to 25° C. is satisfactory for the development of the oak wilt fungus. Most fungi, including *E. fagacearum,* grow slowly on the phenylalanine medium. This is an advantage, for the mycelium may be examined directly with the low power objective of the compound microscope. Often it is helpful to remove the oak chip from the surrounding mycelium before examination.

Identifying Characteristics

On this medium the mycelium of the oak wilt fungus usually appears around the chips after three to five days. It is almost entirely submerged in the agar and is nearly transparent (Fig. 4). It often is necessary to hold the plates up toward the light in order to detect the

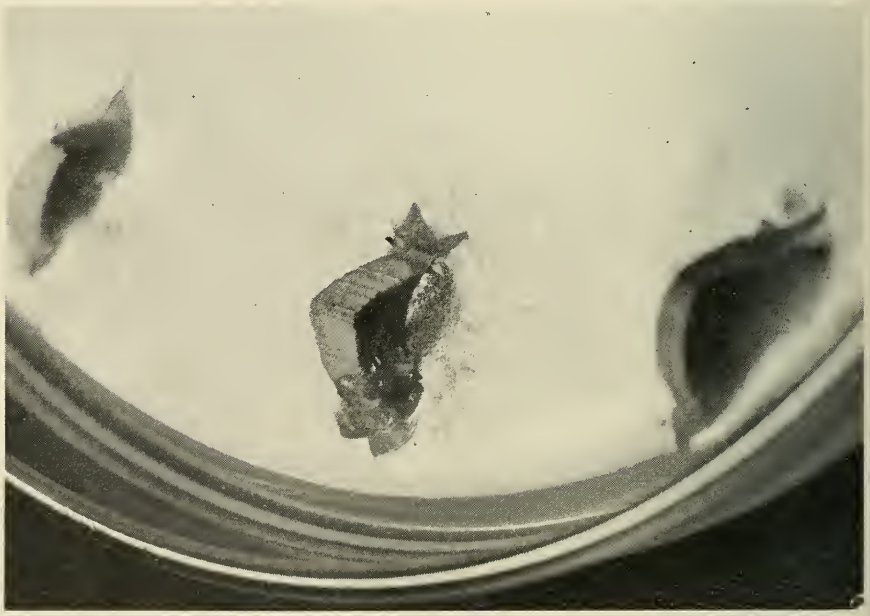

FIGURE 4. THE APPEARANCE of the oak wilt fungus approximately five days after chips are placed on glucose-phenylalanine agar. Note the sparse, submerged mycelium which is scarcely visible at this time.

mycelium. After some experience the observer may be able to recognize the oak wilt fungus on the plates by its characteristic macroscopic appearance. Most other fungi grow more rapidly or more compactly. Macroscopic appearance, however, is seldom enough for accurate diagnosis.

The first-formed conidiophores and conidia are sometimes on the surface but often completely submerged in the agar. The conidiophores are usually formed in loose clusters of three or more and frequently are produced only on certain portions of the mycelium (Fig. 5). The presence of typical conidiophores and endoconidia is usually considered as positive identification of the oak wilt fungus, but, since other endoconidia-producing fungi have been isolated from oak, some caution must be used. The mycelium develops into dark, rather slow growing colonies (Fig. 6) which usually are easy to recognize.

In addition, the presence of certain unusual hyphae on young mycelium growing from oak chips on this medium is believed to be an important diagnostic character (Figs. 7-10). These hyphae are finer than those of the main mycelium or the growing tips. The branches frequently curve or curl back giving a wavy appearance (Figs. 8 and 10).

FIGURE 5. CONIDIOPHORES and clusters of endoconidia produced on the surface of the agar.

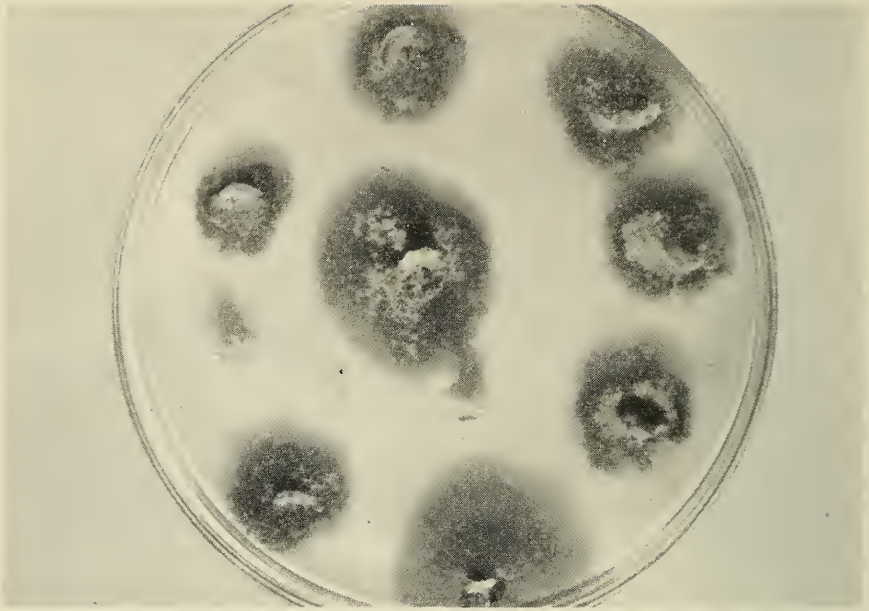

FIGURE 6. OAK WILT FUNGUS growing from oak chips. Age nine days.

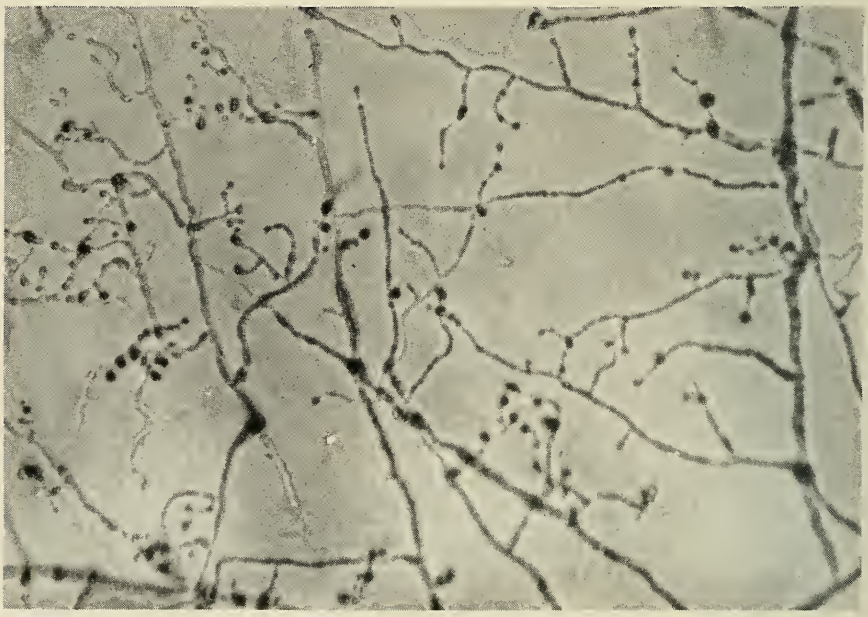

FIGURE 7. LOW POWER VIEW of the characteristic excessively branched hyphae formed on young mycelium from oak chips (unstained preparation).

FIGURE 8. CHARACTERISTIC hyphae enlarged showing manner of branching (unstained preparation).

FIGURE 9. HYPHAE showing two functional conidiophores and two sterile branches (unstained preparation).

FIGURE 10. CLUSTER of functional conidiophores and sterile branches showing the close similiarity between the points of origin and suggesting that the sterile branches are abortive conidiophores.

They are more abundant on some isolates than on others. No other fungus isolated from oak has been seen to produce hyphae similar to these.

Figures 9 and 10 show hyphae on which some of the branches have produced typical conidiophores and conidia while others have remained sterile. It appears that these sterile branched hyphae represent abortive conidiophores, which for some reason have failed to develop normally.

The use of these hyphae as a diagnostic character has made the determination of oak wilt quicker and easier. In our laboratory the cultures are checked first for the presence of these characteristic hyphae and second for typical conidiophores and conidia. In most cases conidia are present, but they often are few and difficult to locate.

This method of isolation and identification of the oak wilt fungus (based on quick sporulation and characteristic branches) has been used with excellent results in our oak wilt research laboratory for the past two years. Positive identification has been made frequently as early as three days, and seldom requires longer than five days. The advantages of this method far outweigh the time and slight inconvenience involved in the preparation of the special glucose-phenylalanine agar medium.

Summary

The medium and methods used successfully for isolation and quick identification of the oak wilt fungus for the last two years are described. A special semisynthetic glucose-phenylalanine agar is favorable for early sporulation. The young mycelium growing from oak chips on agar plates may be examined directly with the compound microscope. The presence of characteristic branched hyphae that appear to be abortive conidiophores is believed to be a dependable diagnostic character. The use of this method usually permits positive determination of oak wilt within three to five days after the oak chips are cultured. This technique should be of value to the workers in many phases of oak wilt research.